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14. ABSTRACT

Our preliminary data on RBC deformability, Ca++ influx and NO production suggest that the increased complement deposition on RBCs in patients with trauma may lead to the cytoskeletal changes in RBCs. This mechanism may retard the ability of RBCs to travel through capillaries and subsequently affect the delivery of O_2 to the tissues. Thus understanding the pathophysiological relevance of complement activation and red cell dysfunction after trauma will provide a rationale to design pharmacological strategies that can prevent complement-RBC mediated post-traumatic mortality.

15. SUBJECT TERMS
RBC, complement deposition, trauma, red cell dysfunction, post-traumatic mortality

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Introduction

Trauma-induced changes in the red blood cells (RBC) contribute to the reduction of blood flow to distant organs. Complement activation has been implicated to mediate early posttraumatic inflammatory response, which leads to the trauma-induced sequelae and adverse outcome.

Erythrocytes (red blood cells, red cells) are the most abundant specialized cells in the human body. During the life cycle of about 120 days, erythrocytes have to maintain their biconcave shape despite regularly passing through capillaries with diameters (4.5-5µm) about half their size (7.4-8µm) [1]. Erythrocyte are able to do this, because their membrane is highly elastic, being about 100 times softer than a latex membrane of comparable thickness, and yet strong enough to undergo rapid and significant shear stresses without fragmentation [2]. Erythrocyte membrane has there major components: **1)** *membrane proteins*, that are either transmembrane or attached to the plasma membrane through GPI- or lipid-anchors (glycophorins, CD47, CR1, band 3, CD55, CD59, flotillin, stomatin etc.) **2)** *skeletal proteins*, located below the plasma membrane, conferring the erythrocyte its specific biconcave shape (spectrin, protein 4.1R, actin) and **3)** *anchoring proteins*, such as ankyrin, tropomyosin, tropomodulin, protein 4.2, adducin, dematin, that connect the membrane with the skeleton beneath, by linking the cytosolic domain of band 3 and glycophorin C with spectrin skeleton [3]. More recently, adducin and dematin have also been implicated in linking plasma membrane protein Glut-1 (glucose transporter-1) to spectrin [4]. Apart from actin, all skeleton and anchoring proteins are phospho-proteins. Importantly phosphorylation status of several skeletal proteins (adducin, ß-spectrin, protein 4.1) was shown to be altered in pathological situations [5-7] [8]. Spectrin tetramers form a hexagonal network (corrals) connected in junctional complexes by protein band 4.1R, adducin (α and β), dematin, tropomodulin and short actin protofilaments [9,10]. These are dynamic micro-domains in erythrocyte membrane that depend on the phosphorylation status of skeletal proteins (see above) and confine the lateral diffusion of transmembrane proteins by slowing down their movement by forcing them to “hop” from one corral to another [11]. We have recently shown that complement-mediated increased confinement by spectrin skeleton of complement regulatory protein CD55 can adversely affect red cell biological functions [12].

Our group and others have shown that CR1 is part of the membrane-bound complement regulatory protein family and is the receptor for all complement opsonins: C3b, C4b, C1q and MBL [13-19]. CR1 along with soluble factor H, control complement activation by degrading C3b to C3d and C3dg. Importantly, most mammals, notably rodents, do not express CR1 and do not use erythrocytes for binding and transporting complement fragments but rather use platelet-adherent factor H to bind immune complexes, which then will be removed along with the carrying platelet, by macrophages in the liver and spleen. Only humans have a transmembrane form of CR1, which makes the human clearance system and the red cell bound complement regulatory system both unique and difficult to model in rodents. We have shown that upon ligation by complement fragments, CR1 actively clusters on the surface of red cells and interacts with a newly described protein phosphatase, FAP-1[20]. The importance of CR1 presence on stored red cells up

until the moment of transfusion is underscored by the significant improvement (over 2 times) of the half-life of human red cells transfused in mice that expressed high titers of anti- human red cells antibodies when soluble CR1 (sCR1) was present along with transfused cells. In addition, the levels of complement fragments deposited on red cells were over 100 times below those of the control mice that were not treated with sCR1[21].

Erythrocyte deformability or elasticity represents the ability of erythrocytes to change their shape in response to an external force and then to return to the original biconcave shape once the force ceases to act upon them. Erythrocyte membrane deformability is one of the most important rheological factors for controlling microcirculation in organs in both normal and ischemic situations [22-24]. The dynamic, energy-dependent linkage between the membrane and the skeleton is paramount for the ability of erythrocytes to squeeze through capillaries [25,26 27]. During blood storage red cell membrane deformability is progressively lost [28-31].

Reactive oxygen species (ROS) represent a collection of molecules or ions formed by the incomplete one-electron reduction of oxygen. This category includes: singlet oxygen; superoxides; peroxides; hydroxyl radical, nitric oxide and hypochlorous acid. Depending on the amount generated, reactive oxygen species can either: **1)** signal in a variety of cellular processes functioning as mediators or **2)** can have a deleterious effect by interacting with a variety of easily oxidizable cellular targets such DNA (mostly deoxyguanosine), proteins, cholesterol and relevant for our proposed studies, with nitric oxide to generate peroxynitrites [32]. Once generated either intracellulary or extra-cellularly, ROS will affect the red cell membrane significantly decreasing its deformability [33-35].

Red blood cells in trauma patients travel through capillaries with difficulty and display poor gas exchange rate and complement fragments deposited on their surface represents the main culprit. We hypothesize that the activated complement fragments deposit on RBC surface and alters their ability to pass through capillaries and exchange gases. This study aims to determine whether complement activation affects erythrocyte physiology in patients with trauma. To understand red cell dysfunction mediated by complement activation, we used both whole RBCs and sera from trauma patients and compared them with the controls.

Body

The clinical significance of trauma cannot be underestimated with over 10 million car accidents occurring and over 35 thousand people dying each year in the U.S. Trauma resulting from accidents or unintentional injuries is the first leading cause of death for those under 50 years old and accounts for one out of about every 20 deaths in the U.S.

Although trauma usually involves a certain anatomical injury involving extremities or the torso and is followed by obvious pathophysiological events usually linked to blood loss, it is invariably associated with manifestations from organs not directly affected by injury. The origin of these secondary manifestations is poorly understood. There is information suggesting the presence of an acute inflammatory response in patients experiencing

trauma. Specifically, there is evidence that complement system is activated and mediates early post-traumatic inflammatory response. In addition, the levels of proinflammatory cytokines, including TNF-á, IL-1 and IL-6 are increased in trauma. It is suggested that complement activation and proinflammatory cytokine storm occurring in patients with trauma collectively choreograph systemic inflammatory response syndrome associated with acute respiratory distress syndrome and multiple organ failure.

In other situations where complement is profusely activated, such as in systemic lupus erythematosus (SLE), complement fragments deposit on the surface of red blood cells (RBC), which limits their deformability while promoting nitric oxide (NO) production. It has been reported that RBC from patients suffering from hemorrhagic shock displays reduced deformability, show altered morphological appearance, and contribute to the reduction of blood flow to distant organs.

Based on the evolving studies, we hypothesized that complement activation affects RBC function in trauma patients. In this report we demonstrate that complement becomes activated in trauma patients and split products are deposited on the surface of RBC limiting their ability to pass through capillary-size microchannels and increasing their capacity to produce NO. Our findings suggest that the inhibition of complement can serve as an adjunct treatment in trauma patients.

Key Research Accomplishments

1. Increased C4d deposition on red blood cell (RBC) in Trauma Patients

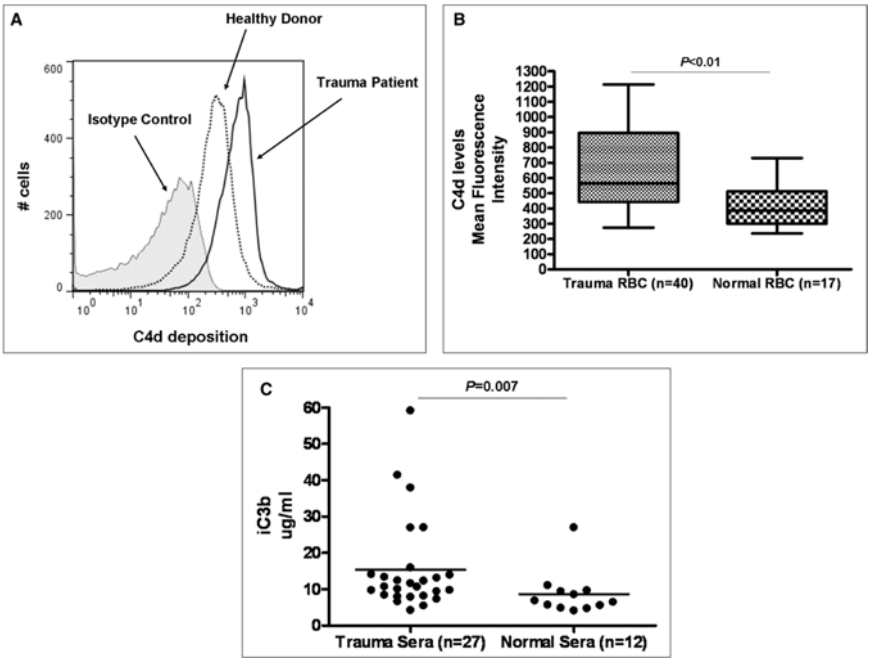

Figure 1. Increased C4d deposition on the surface of membrane of RBCs from trauma. A, Deposition of C4d on the surface of RBCs from trauma patients and healthy donors was measured by flow cytometry. Representative of experiments is shown. B, Data of C4d deposition on RBCs from all samples were expressed as mean fluorescence intensity, and cumulative data from trauma patients (n = 40) or normal healthy donors (n = 17) are shown as box plots. Each box shows the 25th

and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median. C, Serum levels of iC3b fragments from trauma patients (n = 27) and from normal healthy donors (n = 12) were assayed as a measure of activation of complement. Horizontal lines indicate the median.

2. Trauma sera promote C4d deposition on RBC membranes.

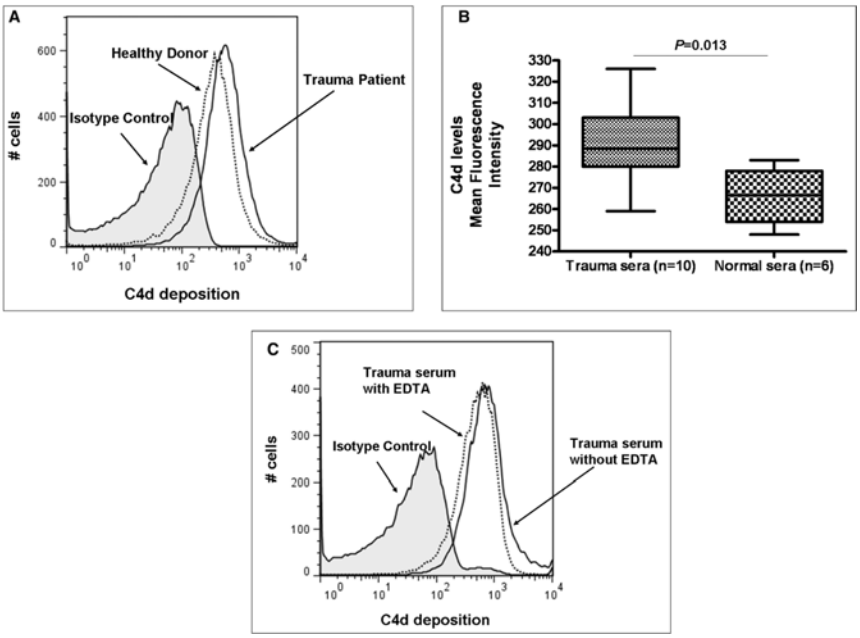

Figure 2. Increased C4d deposition on healthy RBC membranes incubated with trauma sera. A, Deposition of C4d on the surface of healthy RBCs (type O, Rh negative) incubated with sera from trauma patients or healthy donors was measured by flow cytometry. Representative data are shown. B, Data of C4d deposition on RBCs from all samples were expressed as mean fluorescence intensity, and cumulative data from trauma patients (n = 10) or healthy donors (n = 6) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median. C, Deposition of C4d on the surface of healthy RBCs (type O, Rh negative) incubated with sera from trauma patients with or without 10 mM EDTA. Representative data are shown.

3. Trauma sera decrease RBC membrane deformability.

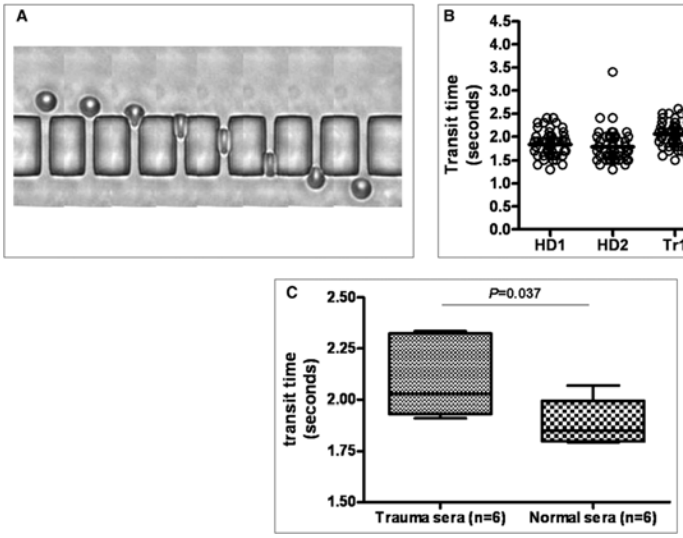

Figure 3. Decreased RBC membrane deformability after incubation with trauma sera. A, A composite series of images illustrating the passage of a single RBC through a capillary-size microchannel of the two-dimensional filter

device. B, Healthy universal donor's (type O, Rh negative) RBCs
deformability were measured by using the two-dimensional filter device after incubation with sera from trauma patients or healthy donors. Decreased deformability of each RBC was associated with increased transit time (i.e., the time it took the cell to pass through the capillary microchannels of twodimensional filter). Data are shown as dot blots of all experiments with each circle showing the passage time for one RBC. Horizontal lines show the mean. Representative data are shown. C, Cumulative data from trauma patients (n = 6) or healthy donors (n = 6) are shown. Data are expressed as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.

4. Phosphorylation status of band 3 in RBCs incubated with trauma sera.

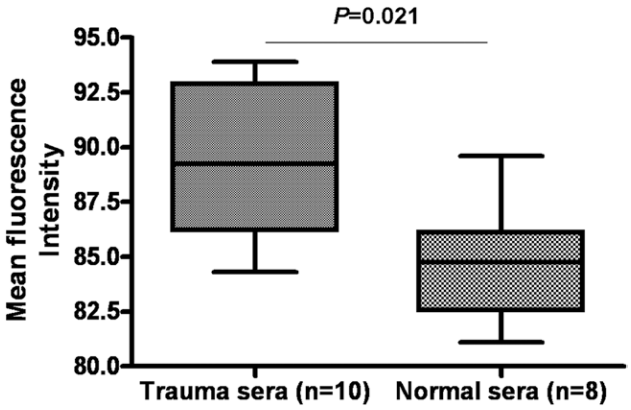

Figure 4. Phosphorylation status of band 3 in RBCs incubated with trauma sera. Phosphorylation of band 3 in healthy RBCs (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors, using eosin-5-maleimide staining. Band 3 phosphorylation was expressed as mean fluorescence intensity of eosin-5-maleimide fluorescence, and cumulative data from trauma patients (n = 10) or healthy donors (n = 8) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.

5. Sera from trauma patients trigger Ca++ influx in RBCs from healthy donors

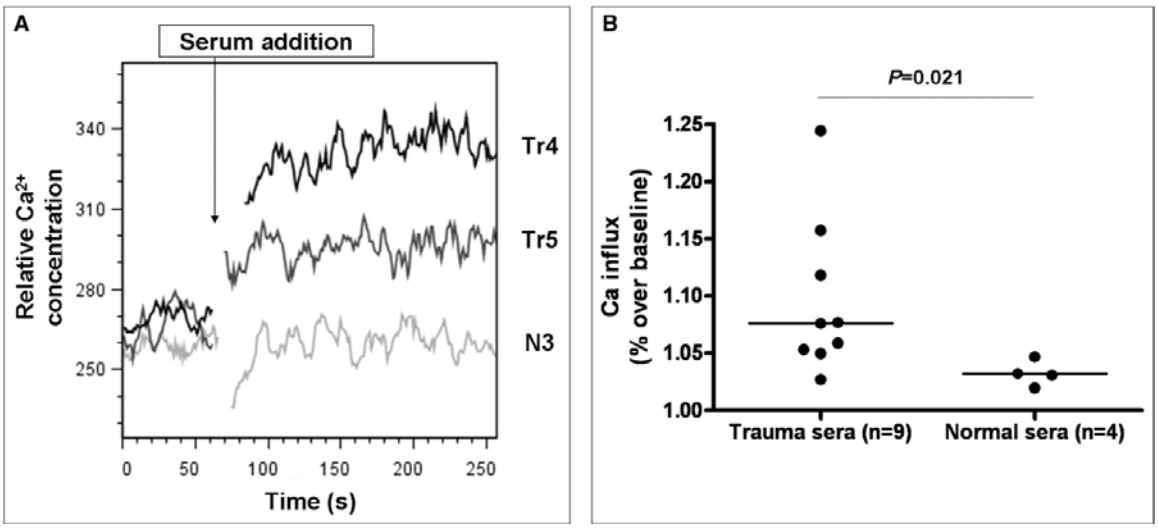

Figure 5. Ca2+ influx into RBC after addition of trauma sera. A, Time course of serum-induced changes in intracellular Ca2+ of trauma patients (Tr4 and Tr5) or a healthy control (N3) are shown. Arrow indicates the time when trauma or control sera were added to RBC preloaded with Fluo-4 AM, 1 min after start of measurement by flow cytometry. Vertical axis indicates relative intracellular Ca2+ concentration, which is estimated from mean fluorescence intensity of Fluo-4 AM. Representative data are shown. B, Cumulative results of Ca2+ influx in RBCs to which trauma sera (n = 9) or normal sera (n = 4) were added. Vertical axis shows the ratio of mean fluorescence intensity of Fluo-4 AM before and after adding the serum. Horizontal lines indicate the median.

6. Trauma serum induced the production of NO in RBCs from healthy donors

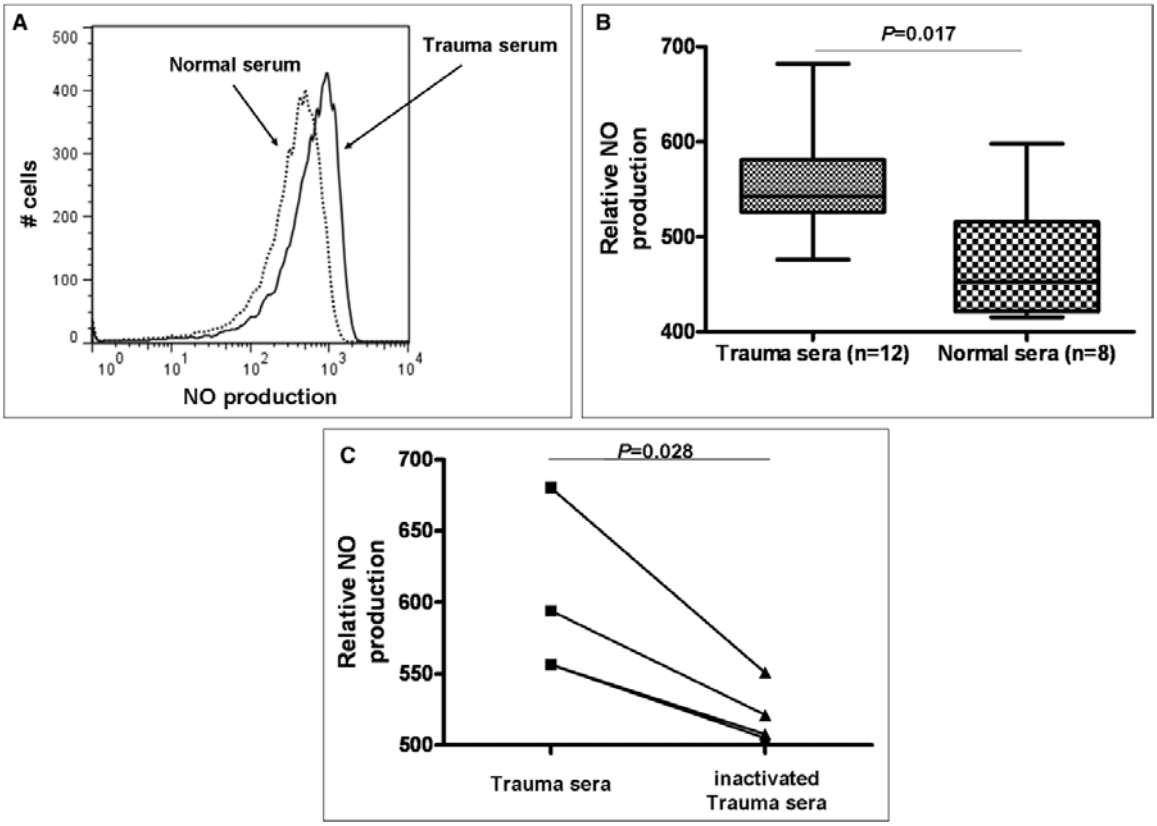

Figure 6. Nitric oxide (NO) production from RBCs induced by trauma serum. A, NO production from healthy RBCs (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors by using DAF-FM diacetate. Representative data are shown. B, NO production from RBCs was expressed as mean fluorescence intensity of DAF-FM diacetate fluorescence, and cumulative data from trauma patients (n = 12) or healthy donors (n = 8) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median. C, Heat inactivation of complement in sera from trauma patients eliminates their ability to trigger NO production. To inactivate complement, sera were incubated 55°C for 30 min. RBCs were incubated with sera from trauma patients before or after inactivation of complement. Individual sera with/without inactivation of complement are shown. Straight lines are used to connect individual points to help visualize how different they are in each sample (n = 4).

Reportable Outcome

Manuscript published.

C4d Deposits on the Surface of Red Blood Cells in Trauma Patients and Interferes with their Function.
Muroya T, Kannan L, Ghiran IC, Shevkoplyas SS, Paz Z, Tsokos M, Lucca JJ, Shapiro

NI, Tsokos GC.
Critical Care Medicine. 2014 Jan 20.

Conclusion

We conclude that C4d decorates the surface of RBC and possibly limits their ability to deform and pass through capillary-size microchannels and increases the production of NO. Thus it may contribute to trauma-associated morbidity and mortality. As a next step, we plan to conduct a prospective study with larger population, which includes clinically homogenous trauma patients compared to matched controls. We believe that our present and future findings might suggest modalities that limit complement activation in trauma patients to be considered for clinical trials.

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Appendices

None.

Supporting data

Included in body.